NOVEL CELL-BASED ASSAYS FOR G-PROTEIN-COUPLED RECEPTOR-MEDIATED ACTIVITIES

INVENTOR(S): Yong YAO, Liang CAO

This application claims priority of provisional application No. 60/330,663, filed October 26, 2001, which is hereby incorporated by reference in its entirety and for all purposes.

FIELD OF THE INVENTION

The invention relates generally to cellular physiology. In particular, the invention relates to materials and methods to identify ligands for G-protein-coupled receptors (GPCRs) and agents that modulate GPCR-mediated activities.

BACKGROUND OF THE INVENTION

G-protein-coupled receptors (GPCRs) comprise a large super-family of integral membrane proteins characterized by having 7 hydrophobic alpha helical transmembrane (TM) domains with three intracellular and three extracellular loops (Ji, *et al.*, *J Biol Chem* 273:17299-17302, 1998). In addition all GPCRs contain N-terminal extracellular and C-terminal intracellular domains. Binding of extracellular ligand may be mediated by the transmembrane domains, the N-terminus, or extracellular loops, either in alone or in combination. For example binding of biogenic amines such as epinephrine, norepinephrine, dopamine, and histamine is thought to occur primarily at the TM3 site while TM5 and TM6 provide the sites for generating an intracellular signal. Agonist binding to GPCRs results in activation of one or more intracellular heterotrimeric GTP-binding proteins (G proteins) which, in turn, transduce and amplify the signal by subsequent modulation of down-stream effector molecules (such as enzymes, ion channels and transporters). This in turn results in rapid production of second messengers (such as cAMP, cGMP, inositol phosphates, diacylglycerol, cytosolic ions).

GPCRs mediate signal transduction across a cell membrane upon the binding of a ligand to a GPCR. The intracellular portion of the GPCR interacts with a G protein to modulate signal transduction from outside to inside a cell. A GPCR is thus coupled to a G protein. There are three polypeptide subunits in a G-protein complex: an alpha subunit–which binds and hydrolyzes GTP–and a dimeric beta-gamma subunit. In the inactive state,

the G protein exists as a heterotrimer of the alpha and beta-gamma subunits. When the G protein is inactive, guanosine diphosphate (GDP) is associated with the alpha subunit of the G protein. When a GPCR is bound and activated by a ligand, the GPCR binds to the G-protein heterotrimer and decreases the affinity of the G alpha subunit for GDP. In its active state, the G subunit exchanges GDP for guanine triphosphate (GTP) and active G alpha subunit disassociates from both the GPCR and the dimeric beta-gamma subunit. The disassociated, active G alpha subunit transduces signals to effectors that are "downstream" in the G-protein signaling pathway within the cell. Eventually, the G protein's endogenous GTPase activity returns active G subunit to its inactive state, in which it is associated with GDP and the dimeric beta-gamma subunit.

The transduction of the signal results in the production of second messenger molecules. Once produced, the second messengers have a wide variety of effects on cellular activities. One such activity is the activation of cyclic nucleotide-gated (CNG) channels by the cyclic nucleotides cAMP and cGMP. CNG channels are membrane spanning molecules that control the flux of cations through the cellular membrane. The channels are activated–opened–by increased intracellular concentrations of cyclic nucleotide. Once opened the channels conduct mixed cation currents, including ions of Na^+, K^+, Mg^{2+} and Ca^{2+}, for example. The activity of the CNG channels couples electrical excitation and Ca^{2+} signaling to changes in the intracellular concentration of cyclic nucleotides (Figure 1).

Receptor function is regulated by the G protein itself (GTP-bound form is required for coupling), by phosphorylation (by G-protein-coupled receptor kinases or GRKs) and by binding to inhibitory proteins known as β-arrestins (Lefkowitz, *J Biol Chem*, 273:18677-18680, 1998). It has long been established that many medically significant biological processes are mediated by proteins participating in signal transduction pathways that involve G proteins and/or second messengers (Lefkowitz, *Nature*, 351:353-354, 1991). In fact, nearly one-third of all prescription drugs are GPCR ligands (Kallal *et al.*, *Trends Pharmacol Sci*, 21:175-180, 2000).

GPCRs fall into three major classes (and multiple subclasses) based on their known (or predicted) structural and functional properties (Rana *et al.*, *Ann Rev Pharmacol Toxicol*, 41:593-624, 2001; Marchese *et al.*, *Trends Pharmacol Sci*, 20:370-375, 1999). Most of these receptors fall into class A, including receptors for odorants, light, and biogenic amines, for chemokines and small peptides, and for several glycopeptide/glycoprotein hormones. Class B receptors bind higher molecular weight hormones while class C includes $GABA_B$

receptors, taste receptors, and Ca^{2+}-sensing receptors. GPCRs are found in all tissues. However, expression of any individual receptor may be limited and tissue-specific. As such some GPCRs may be used as markers for specific tissue types.

As might be expected from the wide range of GPCRs and GPCR ligands, aberrant function of these molecules has been implicated in a large number of human disease states (Rana *et al.* and Ji *et al.*, supra). GPCR agonists and antagonists have been developed to treat many of these diseases. For example the important group of receptors for biogenic amines has been the target of a large number of successful drugs. Among the receptors in this group are those for epinephrine and norepinephrine (α- and β-adrenergic receptors), dopamine, histamine, and serotonin. Examples of diseases in which GPCR function has been implicated include, but are by no means limited to: heart disease (*e.g.* tachycardia, congestive heart failure, etc.), asthma, hypertension, allergic reactions (including anaphylactic shock), gastrointestinal disorders, and a wide range of neurological disorders (*e.g.* Parkinson's disease, depression, schizophrenia, etc.). Finally, many receptors for drugs of abuse are GPCRs.

In many animals, GPCRs are found throughout the organism and are responsible for the maintenance of normal function as well as for pathological conditions. In other instances, the expression of specific GPCRs or families of GPCRs is very tightly controlled, *e.g.*, being expressed only during early developmental stages, etc. Consequently, it is important to find compounds that can stimulate or activate GPCRs, or inhibit or deactivate GPCRs as needed. Agonists–compounds that stimulate the normal function of the GPCRs–have been used to treat asthma, Parkinson's disease, acute heart failure, osteoporosis, hypotension, etc. Antagonists, compounds that interfere with or block normal function have been used to treat, hypertension, myocardial infarction, ulcers, asthma, allergies, psychiatric and neurological disorders, anorexia and bulimia.

In addition to well-characterized receptors, many "orphan" receptors have been cloned (Marchese *et al.*, supra) which are known from sequence similarities to be part of these families, but for which no function or ligand(s) have been discerned. Given the central role of GPCRs in control of diverse cellular activities, there remains a need in the art for methods to identify the agonists and antagonists of these "orphan" receptors as well as to identify additional antagonists for those receptors whose agonists–ligands–are known.

As the first recognized second messenger, cAMP is synthesized by adenylate cyclase in response to activation of many receptors coupled to G proteins G_s and G_{olf} and cyclase

activity is inhibited by activation of receptors coupled to G protein G_i. cAMP activates cAMP-dependent protein kinase A (PKA) resulting in profound cellular responses. Physiologically, cAMP mediates such hormonal responses as mobilization of stored energy (*e.g.*, the breakdown of carbohydrates in liver or triglycerides in fat cells, conservation of water by kidney, and Ca^{2+} homeostasis), control of the rate and contraction force of the heart muscle, relaxation of smooth muscle, production of sex hormones, and many other endocrine and neural processes.

There are a number of cAMP assays currently available. They include transcription reporter assay where a luciferase reporter is driven with a cAMP response promoter element CRE, cAMP immunoassay (Applied Biosystems Forster City, CA), an *in vitro* enzymatic assay for adenylyl cyclase (Molecular Devices, Sunnyvale, CA) and cAMP fluorescence polarization assay (PerkinElmer Life Sciences, Boston, MA). However, all these assays are end point assays where the cells are lysed and extracts are used for the tests. R.Y. Tsien and his colleagues have also developed fluorescent probes that report cAMP levels in single cells. However, the methods of application of these probes to cells makes them not suitable for high throughput screening formats (Adams *et al.*, 1991, Nature 349:694-697; Zoccolo *et al.*, 2000, Nat. Cell Biol. 2:25-29). There is a need in the art to be able to detect the activation of individual living cells for their cAMP production, particularly in a heterogeneous cell or tissue environment. Such detection capability would further allow the examination of receptor activation and cellular response to complex stimuli, as in the case of induced long-term memory. There also exists in the art a need for the ability to directly examine the cAMP in live cells in order to identify ligands for orphan GPCRs based on the concurrent examination of both Ca^{2+} and cAMP activation in a given cell as well as to identify agents that modulate GPCR-mediated activity. These and other needs are met by the present invention.

SUMMARY OF THE INVENTION

The assays and methods of the present invention utilize CNG channels to monitor the activity of GPCR signaling cascades, in particular the activity of GPCRs. In these assays the GPCRs and CNG channels may be endogenous to the cells or may be exogenously supplied. In addition, endogenous or exogenously supplied G proteins, including promiscuous G proteins, may be used in the assays and methods of the invention.

In some embodiments, the present invention provides a host cell that contains a first nucleic acid comprising a first promoter operably linked to a first polynucleotide wherein the polynucleotide comprises a sequence encoding a G protein-coupled receptor (GPCR) protein and a second nucleic acid comprising a promoter operably linked to a second polynucleotide wherein the second polynucleotide comprises a sequence encoding a cyclic nucleotide-gated (CNG) channel. In some embodiments, the cyclic nucleotide-gated channel comprises at least one mutation that makes the channel more sensitive to cAMP than a channel that does not comprise the mutation. In some embodiments, the GPCR and/or the CNG channel is not normally expressed in the cell. The nucleic acids may be part of one molecule or may be parts of different molecules. The nucleic acids may be provided to the cell in any formulation known to those skilled in the art, for example, one or both of the nucleic acids may be part of a virus and/or plasmid and/or may be expressed from the genome of the cell.

In some embodiments, it may be desirable to utilize or create a cell line that expresses one or more of the molecules from the genome of the cell. The creation of stable cell lines for the expression of proteins is within the capability of one ordinarily skilled in the art. Some embodiments of the present invention may include expressing one protein from the genome of the cell and the other from an exogenous nucleic acid, preferably a virus or a plasmid. Cells of the present invention may be any kind of cell but are preferably eukaryotic cells such as mammalian cells. Examples of cells suitable for the practice of the present invention include, but are not limited to, BHK cells, mouse L cells, Jurkat cells, 153DG44 cells, HEK cells, CHO cells, PC12 cells, human T-lymphocyte cells and Cos-7 cells.

The CNG channels used in the present invention may be wildtype channels or may be mutated to make them more responsive to cAMP. The wildtype CNG channels of the present invention may be homomeric or heteromeric. The channels may comprise one or more mutations that make the channel more sensitive to cAMP than a channel that does not comprise the mutations. Channels that comprise two or more mutations that make the channel more sensitive to cAMP than a channel that does not comprise the mutations are also included in the present invention. Channels that comprise three or more mutations that make the channel more sensitive to cAMP than a channel that does not comprise the mutations are also included in the present invention. Nucleic acid molecules encoding CNG channels of the invention may comprise all or part of one or more of the nucleic acid sequences provided as SEQ ID NOS:1, 5, and 7. Some CNG channel proteins of the present invention may

comprise all or part of one or more of the protein sequences provided as SEQ ID NOS:2, 4, 6, and 8.

In some embodiments, the CNG channels used in the present invention may be responsive to cGMP. In other embodiments, the CNG channels used in the present invention may be responsive to analog or derivative cyclic purine monophosphates (cPuMP) or cyclic nucleotide monophosphates (cNMP). In still other embodiments, a CNG channel used in the present invention may be responsive to only one of cAMP, cGMP, an analog or derivative cPuMP or a cNMP. In a preferred embodiment, a CNG channel used in the present invention may be responsive to at least one of cAMP, cGMP, an analog or derivative cPuMP, or a cNMP. In yet another preferred embodiment, a CNG channel used in the present invention may be responsive to two or more of cAMP, cGMP, an analog or derivative cPuMP, or a cNMP.

The nucleic acid molecules encoding GPCRs according to the present invention may encode a full length wildtype G protein-coupled receptor or may encode a mutant GPCR. Some preferred mutants include N- and C-terminal truncations and insertion and/or deletion mutants. Other preferred mutants may have at least one conservative or non-conservative amino acid base substitution. Still other preferred mutants may have a combination of mutations, comprising at least two selected from the group consisting of N-terminal truncations, C-terminal truncations, insertions, deletions, conservative amino acid base substitutions and non-conservative amino acid base substitutions. A mutant GPCR is suitable for use in the present invention if it is capable of inducing a GPCR-mediated activity when contacted with an agonist.

In some embodiments, cells of the present invention may contain a third nucleic acid comprising a third promoter operably linked to a third polynucleotide wherein the third polynucleotide comprises a sequence encoding a G protein. The G protein may be a promiscuous G protein. The G protein may be normally expressed in the cell but may be expressed at a higher level when the cell contains the third nucleic acid. Alternatively, the G protein may not be naturally expressed in the cell.

In some embodiments of the invention, the G protein-coupled receptor is substantially coupled to at least one G protein selected from the group consisting of $G\alpha_s$, $G\alpha_i$, $G\alpha_{16}$ or $G\alpha_q$. and promiscuous G proteins. Alternatively, the G protein-coupled receptor may be substantially coupled to a hybrid G protein, such as $G\alpha_{qs}$, for example.

In another aspect, the present invention provides a method of detecting activity of a GPCR by expressing the GPCR in a cell–optionally from an exogenous GPCR-encoding nucleic acid molecule–expressing a cyclic nucleotide-gated channel that may comprise one or more mutations that make the channel more sensitive to cAMP; and measuring activity of the channel wherein activity of the channel indicates activity of the GPCR. The CNG channel may be expressed from an exogenous nucleic acid or from the genome of the cell. In some embodiments, measuring may entail the use of a dye, for example, a fluorescent dye that can be detected by UV-based imaging systems. Some preferred dyes include, but are not limited to, Ca^{2+} sensitive dyes and voltage sensitive dyes. In some embodiments, measuring may entail determination of activation of CNG channel activity in a single cell. This may be accomplished using any means known to persons skilled in the art such as by UV-based fluorescence using a microscope. When a microscope is used it may be desirable to couple the microscope to a computer system. The computer system may be used to track individual cells and perform statistical analysis.

In some embodiments, the method may be configured to be conducted in a multiwell plate–96 well, 384 well etc.–and measuring may be performed with a multiwell microplate reader. Examples of suitable readers include those that are fluorometric-based readers with a CCD camera and fluorometric-based scanning microplate readers.

In some embodiments, it may be desirable to attach the cells to a solid surface before, during or after performing the methods of the invention. Suitable solid surfaces include, but are not limited to, slides and multiwell plates.

In some instances it may be desirable to increase the sensitivity of the methods of the invention. This may be accomplished by, for example, pretreating the cells with a cAMP analogue before measuring. Suitable analogues include caged photoactivatable analogues.

The methods of the invention may be practiced with cells expressing a promiscuous G protein. The promiscuous G protein may be expressed from the genome of the cell and/or may be expressed from an exogenous nucleic acid.

In some embodiments of the invention, the GPCR-mediated activity to be measured may be ion flux. In these cases, ion flux may be measured by any method known to those skilled in the art including, but not limited to, by determining a change in spectral characteristic of a dye or by patch clamp.

In another aspect, the present invention provides a method of identifying a ligand for a receptor by contacting a cell with a compound wherein the cell expresses the receptor and at

least one cyclic nucleotide-gated (CNG) channel and measuring activation of the CNG channel, wherein activation of the CNG channel indicates that the compound is a ligand for the receptor. In some embodiments, the receptor may not be endogenous to the cell and/or the CNG channel may be engineered to increase the channel sensitivity to cAMP. The CNG and/or the GPCR channel may be expressed from an exogenous nucleic acid and/or from the genome of the cell. Measuring of the CNG channel activation may be by any means known to those skilled in the art including , but not limited to, by the use of a dye. An example of a suitable dye is a fluorescent dye that can be detected by UV-based imaging systems. Preferably a dye may be a Ca^{2+} sensitive dye and/or a voltage sensitive dye.

The methods of identifying a ligand may be used on a single cell by measuring activation of CNG channel activity in a single cell. Methods of this type may employ the use of UV-based fluorescence detection using a microscope. When a microscope is used, it may be desirable to couple the microscope to a computer system. The computer system may be used to track individual cells and perform statistical analysis.

The methods of identifying a ligand may be used in a multiwell–96 well, 384 well, etc–format and measuring may be performed with a microplate reader. A suitable reader may be a fluorometric-based reader with a CCD camera and/or a fluorometric-based scanning microplate reader.

The methods of identifying a ligand may be used with cells attached to a solid surface. The cells may be attached before, during or after performing one or more of the method steps. Suitable solid surfaces include, but are not limited to, slides and multiwell plates.

The methods of identifying a ligand may be used with cells that have been pretreated with a cAMP analogue before being contacted with the ligand, for example, with a caged, photoactivatable analogue.

The methods of identifying a ligand may be used with one or more cells that express a promiscuous G protein.

In some embodiments, the methods of identifying a ligand may include a measuring step that comprises determining ion flux. Ion flux may be determined by any means known to those skilled in the art such as by a change in spectral characteristic of a dye and/or by patch clamp.

In another aspect, the present invention provides a method of identifying an agent that modulates an activity mediated by a GPC receptor by contacting a cell with the agent and a ligand for the receptor wherein the cell expresses the receptor and at least one cyclic

nucleotide-gated (CNG) channel including wildtype or CNGs engineered to increase the channel sensitivity to cAMP and measuring activation of the CNG channel. In some embodiments, it may be desirable to compare activation of the CNG channel in the presence of the agent to activation of the channel in the absence of the agent. Typically, a difference in activation of the CNG channel indicates the agent modulates the activity. The CNG channel may be expressed from an exogenous nucleic acid and/or from the genome of the cell. Measuring the activation of the CNG channel may entail the use of a dye. An example of a suitable dye is one that is a fluorescent dye that can be detected by UV-based imaging systems. Dyes may be Ca^{2+} sensitive dyes and/or voltage sensitive dyes. Dyes of the present invention may be added exogenously to the cells either before or during the assay. Alternatively, dyes of the present invention may be expressed exogenously by the cells as probes. Said probes may be introduced into said cells for transient expression or for stable expression.

The methods of identifying an agent that modulates an activity mediated by a GPC receptor may be practiced on a single cell by determination of activation of CNG channel activity in a single cell. Methods of making such a determination are known to those skilled in the art and include by UV-based fluorescence using a microscope. When a microscope is used it may be coupled to a computer system. The computer system may be one that tracks individual cells and performs statistical analysis.

The methods of identifying an agent that modulates an activity mediated by a GPC receptor may be configured to use a multiwell–96 well, 384 well etc–format. Configurations of this type may employ a multiwell microplate reader, for example, a fluorometric-based reader with a CCD camera and/or a fluorometric-based scanning microplate reader.

The methods of identifying an agent that modulates an activity mediated by a GPC receptor may be practiced on cells attached to a solid support. The cells may be attached before, during or after performing one or more method steps. Suitable solid supports include slides and multiwell plates.

The methods of identifying an agent that modulates an activity mediated by a GPC receptor may be performed using cells pretreated with a cyclic nucleotide analogue. Suitable analogues include caged, photoactivatable analogues.

Any of the methods of identifying an agent that modulates an activity mediated by a GPC receptor may be practiced using cells that express a promiscuous G protein.

The present invention further provides kits adapted to perform the methods of the invention. Such kits will typically include one or more cells of the invention in a suitable container. Kits may optionally comprise one or more reagents such as buffers and/or salts and/or dyes. When dyes are included, they will typically be voltage sensitive dyes and/or Ca^{2+} sensitive dyes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic of the signaling pathway from a GPCR to a CNG channel.

Figure 2A is a photograph of HEK239 cells transfected with a mutated rat CNG channel gene (SEQ ID NO 3) prior to their being stimulated with a ligand for an endogenous GPCR. Figure 2B shows the cells after stimulation with norepinephrine (NE) that activates endogenous β2-adrenoceptor, a Gs coupled receptor. The signal is detected with a calcium sensitive fluorescence dye, Fura-2 (Molecular Probes, Eugene, OR) by using a fluorescence microscope. Figure 2C illustrated an integrated result on intracellular Ca^{2+} concentration of 100 individual cells by using ATTO Graph (ATTO, Rockville, MD) as a function of time. A plasmid expressing a green fluorescence protein (GFP) was co-transfected with the CNG gene in the host cells.

Figure 3A is a graph of intracellular Ca^{2+} concentration as a function of time in cells transfected with a mutated CNG channel gene (SEQ ID NO 5) and stimulated with a ligand to Gs coupled endogenous β2-adrenoceptor that results in the accumulation of cAMP and the activation of the CNG channel. The determination of concentration was made by fluorescence of an intracellular dye (Fura-2) as measured by a commercially available microplate reader. Figure 3B is a graph of intracellular Ca^{2+} concentration as a function of time in cells transfected with a CNG channel and stimulated with a ligand activating a Gq coupled GPCR, resulting in the mobilization of intracellular Ca^{2+} stores.

Figure 4 is a graph of membrane potential as a function of time in cells transfected with a mutated CNG channel gene (SEQ ID NO 7) and stimulated with forskolin, a direct adenylyl cyclase activator, resulting in the activation of the CNG. The determination of membrane potential was made using a commercially available voltage sensitive dye kit in a multiwell plate reader.

Figure 5 is a graph of membrane potential as a function of time in cells transfected with a mutated CNG channel gene (SEQ ID NO 5) and stimulated with a ligand for Gs coupled endogenous β2-adrenoceptor that results in the production of cAMP and activation

of the CNG channel. The determination of membrane potential was made using a commercially available voltage sensitive dye kit in a multiwell plate reader.

Figure 6 is a graph of membrane potential as a function of time in cells transfected with a triple mutant CNG channel gene (SEQ ID NO 7) and stimulated with the indicated concentrations of a ligand to an endogenous Gs coupled β2-adrenoceptor that results in activation of the CNG channel. The determination of membrane potential was made using a commercially available voltage sensitive dye kit in a multiwell plate reader.

Figure 7 is a graph of membrane potential as a function of time in cells transfected with both a mutated CNG channel gene (SEQ ID NO 7) and an exogenous Gs coupled GPCR, dopamine receptor D1, and stimulated with a dopamine. The determination of membrane potential was made using a commercially available voltage sensitive dye kit in a multiwell plate reader.

Figures 8A-C provide a sequence alignment of human CNG channels.

Figures 9A-B provide a sequence alignment of mammalian CNG channels.

Figures 10A-D. Figure 10A depicts the response of HEK293H-CNG cells to 1 μM isoproterenol. Figure 10B depicts the response of non-transformed parental HEK293H cells to 1 μM isoproterenol. Figure 10C depicts dose-response curves of HEK293H-CNG cells to 0 (control), 1 nM, 3 nM, 10 nM, 30 nM, 0.1 μM, 0.3 μM and 1 μM isoproterenol. Figure 10D depicts the well-to-well consistency of the readings for measuring CNG activation.

Figure 11 depicts calcium uptake by HEK293H-CNG cells in a dose-dependent manner to 0 (control), 0.3, 1, 3, 10, 30 and 300 nM isoproterenol.

Figures 12A-B. Figure 12A shows voltage sensitive fluorescence of the same living cells immediately before the addition of 1 μM isoproterenol and 15, 30 and 45 seconds after addition. Figure 12B depicts the background corrected average fluorescence of 71 imaged cells.

Figure 13 depicts the sensitivity of CNG channels which are composed of wildtype heteromeric (α+β) subunits, wildtype homomeric (α) subunits, or mutant homomeric CNG channels comprising one (Y565A), two (C460R/E583M and C460H/E583M), or three (C460W/Y565A/E583M) substitution mutations.

Figure 14 depicts a comparison between the CNG channel assay and conventional CRE and ELISA assays. Each assay format is depicted as a dose-response to forskolin stimulation.

Figure 15 depicts the sensitivity of the CNG channel assay for activation of GPCRs for various ligands. Representative dose-response curves are depicted for Parathyroid Hormone Receptor 1 (PTHR1), Histamine Receptor H2 (HRH2), and 5-Hydroxytryptamine 4 Receptor (5-HT4) response to the agonists PTHrP (peptide), Histamine (monoamine), and 5-HT (monoamine), respectively.

Figure 16 depicts a dose-response comparison of the CNG channel assay using membrane potential dye to calcium assays using promiscuous G protein ($G\alpha_{16}$) or chimeric G protein ($G\alpha_{qs}$) for detecting activation of the tyramine receptor.

Figure 17A depicts the response of HEK293H-CNG cells to a panel adrenergic compounds as depicted in Figure 17B.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. General Description

The present invention provides materials and methods to analyze GPCR-mediated activity. Additionally, the materials and methods of the invention may be used to screen synthetic small molecules and combinatorial or naturally occurring compound libraries to discover novel therapeutics to regulate G-protein signaling.

A. Definitions

In the description that follows, numerous terms and phrases known to those skilled in the art are used. In the interest of clarity and consistency of interpretation, the definitions of certain terms and phrases are provided.

As used herein, "substantially interacts" refers to the amount of an effect one molecule has on another, for example, the effect of a GPCR on a G protein. An interaction is substantial if it results in a detectable response of an amplitude capable of having a physiological effect.

As used herein, the "genome" of a cell refers to the genetic material contained on the chromosomes of the cell.

As used herein, "GPCR-mediated activity" refers to any cellular process that can be affected by signal transduction mediated by a GPCR. This phrase is seen to include, but is not limited to cyclic nucleotide production, Ca^{2+} influx, inositol triphosphate (IP_3) and diacylglycerol production and the like.

In some instances, putative GPCRs may be identified by homology to other known GPCRs. Homology or identity at the nucleotide or amino acid sequence level is determined by **BLAST** (**B**asic **L**ocal **A**lignment **S**earch **T**ool) analysis using the algorithm employed by the programs **blastp, blastn, blastx, tblastn** and **tblastx** (Altschul *et al.*, *Nucleic Acids Res* 25: 3389-3402, 1997 and Karlin *et al.*, *Proc Natl Acad Sci USA* 87:2264-2268, 1990, both fully incorporated by reference) which are tailored for sequence similarity searching. The approach used by the **BLAST** program is first to consider similar segments, with and without gaps, between a query sequence and a database sequence, then to evaluate the statistical significance of all matches that are identified and finally to summarize only those matches which satisfy a preselected threshold of significance. For a discussion of basic issues in similarity searching of sequence databases, see Altschul *et al.* (*Nature Genetics* 6:119-129, 1994) which is fully incorporated by reference. The search parameters for **histogram**, **descriptions**, **alignments**, **expect** (*i.e.*, the statistical significance threshold for reporting matches against database sequences), **cutoff**, **matrix** and **filter** (low complexity) are at the default settings. The default scoring matrix used by **blastp**, **blastx**, **tblastn**, and **tblastx** is the **BLOSUM62** matrix (Henikoff *et al.*, *Proc Natl Acad Sci USA* 89:10915-10919, 1992, fully incorporated by reference), recommended for query sequences over 85 in length (nucleotide bases or amino acids).

For **blastn**, the scoring matrix is set by the ratios of **M** (*i.e.*, the reward score for a pair of matching residues) to **N** (*i.e.*, the penalty score for mismatching residues), wherein the default values for **M** and **N** are +5 and -4, respectively. Four **blastn** parameters were adjusted as follows: Q=10 (gap creation penalty); R=10 (gap extension penalty); wink=1 (generates word hits at every winkth position along the query); and gapw=16 (sets the window width within which gapped alignments are generated). The equivalent **Blastp** parameter settings were Q=9; R=2; wink=1; and gapw=32. A **Bestfit** comparison between sequences, available in the GCG package version 10.0, uses DNA parameters GAP=50 (gap creation penalty) and LEN=3 (gap extension penalty) and the equivalent settings in protein comparisons are GAP=8 and LEN=2.

As used herein, "stringent conditions" are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS at 50°C, or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM

sodium citrate at 42°C. Another example is hybridization in 50% formamide, 5× SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5× Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2× SSC and 0.1% SDS. A skilled artisan can readily determine and vary the stringency conditions appropriately to obtain a clear and detectable hybridization signal. Preferred molecules are those that hybridize under the above conditions to the complement of SEQ ID NO: 1, 3, and which encode a functional protein. Even more preferred hybridizing molecules are those that hybridize under the above conditions to the complement strand of the open reading frame of SEQ ID NO: 1, 3.

As used herein, a nucleic acid molecule is said to be "isolated" when the nucleic acid molecule is substantially separated from contaminant nucleic acid molecules encoding other polypeptides.

The present invention further provides fragments of the encoding nucleic acid molecule. As used herein, a fragment of an encoding nucleic acid molecule refers to a small portion of the entire protein coding sequence. The size of the fragment will be determined by the intended use. For example, if the fragment is chosen so as to encode an active portion of the protein, the fragment will need to be large enough to encode the functional region(s) of the protein. For instance, fragments that encode peptides corresponding to predicted antigenic regions may be prepared. If the fragment is to be used as a nucleic acid probe or PCR primer, then the fragment length is chosen so as to obtain a relatively small number of false positives during probing/priming.

Fragments of the encoding nucleic acid molecules of the present invention (*i.e.*, synthetic oligonucleotides) that are used as probes or specific primers for the polymerase chain reaction (PCR), or to synthesize gene sequences encoding proteins of the invention, can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci *et al.* (*J Am Chem Soc* 103, 3185-3191, 1981) or using automated synthesis methods. In addition, larger DNA segments can readily be prepared by well-known methods, such as synthesis of a group of oligonucleotides that define various modular segments of the gene, followed by ligation of oligonucleotides to build the complete modified gene.

As used herein, "mutant" or "mutated" CNG channels are those comprising subunits that have an altered amino acid sequence. Alterations of the amino acid sequence may include, but are not limited to, N-terminal truncations, C-terminal truncations, amino acid residue deletions or additions, conservative or non-conservative amino acid residue

substitutions. A mutant CNG channel subunit may comprise one or more, two or more or three or more alterations to its amino acid sequence. A mutant CNG channel may be heteromeric, being composed of at least two different subunit types, such as α and β. A mutant CNG channel may be heteromeric in comprising subunits that have different mutations. A mutant CNG channel may be heteromeric in comprising at least one mutant subunit and at least one wildtype subunit. A mutant CNG channel may also be a heteromer comprising subunits corresponding to all of the wildtype subunits it is normally composed of when expressed in its native cell source, wherein at least one of those subunits comprises at least one alteration of its amino acid sequence. A mutant CNG channel may be composed of subunits derived from the same species as the recombinant cell transformed to express the CNG channel. A mutant CNG channel may be composed of subunits derived from a different species as the recombinant cell transformed to express the CNG channel. A mutant CNG channel may be composed of subunits derived different species. Mutant CNG channels may comprise subunits derived from any species, including, but not limited to, rat, murine, human, bovine, canine, feline, any other mammal or vertebrate, Drosophila and other insects, and *C. elegans*, for example.

As used herein, "wildtype" CNG channels are those composed of subunits that have not had mutations made to the amino acid sequence of those subunits as isolated from natural sources or subunits with mutations as compared to the subunit isolated from natural sources, wherein the mutations do not substantially alter channel function or activity. A wildtype CNG channel is preferably heteromeric, being composed of at least two different subunit types, such as α and β. A wildtype CNG channel may also include, in some preferred embodiments, a third different subunit. A wildtype CNG channel may also be a heteromer comprising all of the subunits it is normally composed of when expressed in its native cell source. A wildtype CNG channel may be composed of subunits derived from the same species as the recombinant cell transformed to express the CNG channel. A wildtype CNG channel may be composed of subunits derived from a different species as the recombinant cell transformed to express the CNG channel. A wildtype CNG channel may be composed of subunits derived different species. Wildtype CNG channels may also comprise subunits derived from any species, including, but not limited to, rat, murine, human, bovine, canine, feline, any other mammal or vertebrate, Drosophila and other insects, and *C. elegans*, for example. CNG channel subunits of the present invention may be present on a single or on multiple vectors for introduction into a host cell. For example, in the case of a wildtype

α/β heteromeric CNG channel, the coding sequences for the α and β subunits may be contained on a single vector which is introduced into the host cell, or they may on separate vectors which are introduced into the host cell either separately or at the same time.

As used herein, "voltage sensitive dyes" or "membrane potential dyes" include those dyes that enter depolarized cells, bind to intracellular proteins or membranes and exhibit enhanced fluorescence. Voltage sensitive dyes include, but are not limited to, carbocyanine, rhodamine, oxonols, and merocyanine bis-barbituric acid oxonols. Voltage sensitive and membrane potential dyes also include probes which are encoded by nucleic acid sequences that can be incorporated into a vector for expression by a host cell.

As used herein, "calcium-sensitive dyes" include those dyes which exhibit enhanced fluorescence in response to increased levels of intracellular calcium. Calcium-sensitive dyes include, but are not limited to, Fura-2, Fluo-3, Fluo-4, and Calcium Green-1. Calcium-sensitive dyes also include probes which are encoded by nucleic acid sequences that can be incorporated into a vector for expression by a host cell and include, but are not limited to, Aeuorin (Euroscreen) and green flourescent protein (GFP)-based calcium sensors such as Cameleon, for example.

B. Techniques

The present invention further provides recombinant DNA molecules (rDNAs) that contain a coding sequence. Preferred coding sequences are those that encode wildtype or mutant forms of one or more of GPCRs and/or G proteins and/or CNG channels. As used herein, a rDNA molecule is a DNA molecule that has been subjected to molecular manipulation *in situ*. Methods for generating rDNA molecules are well known in the art, for example, see Sambrook *et al.*, (Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989). In the preferred rDNA molecules, a coding DNA sequence is operably linked to expression control sequences and/or vector sequences.

The choice of vector and/or expression control sequences to which one of the protein encoding sequences of the present invention is operably linked depends directly, as is well known in the art, on the functional properties desired, *e.g.*, protein expression, and the host cell to be transformed. A vector contemplated by the present invention is at least capable of directing the replication or insertion into the host chromosome, and preferably also expression, of the structural gene included in the rDNA molecule.

Expression control elements that are used for regulating the expression of an operably linked protein encoding sequence are known in the art and include, but are not limited to, inducible promoters, constitutive promoters, secretion signals, and other regulatory elements. Preferably, the inducible promoter is readily controlled, such as being responsive to a nutrient in the host cell's medium.

Expression vectors compatible with eukaryotic cells, preferably those compatible with mammalian cells, can be used to form rDNA molecules that contain a coding sequence. Eukaryotic cell expression vectors, including but not limited to viral vectors and plasmids, are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are pSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), and the like eukaryotic expression vectors.

Eukaryotic cell expression vectors used to construct the rDNA molecules used in the present invention may further include a selectable marker that is effective in an eukaryotic cell, preferably a drug resistance selection marker. An example of a drug resistance marker is the gene whose expression results in neomycin resistance, *i.e.*, the neomycin phosphotransferase (*neo*) gene (Southern *et al.*, *J Mol Anal Genet* 1:327-341, 1982). Alternatively, the selectable marker can be present on a separate plasmid, and the two vectors are introduced by co-transfection of the host cell, and selected by culturing in the appropriate drug for the selectable marker.

The nucleic acid molecule is then preferably placed in operable linkage with suitable control sequences, as described above, to form an expression unit containing the protein open reading frame. The expression unit is used to transform a suitable host and the transformed host is cultured under conditions that allow the production of the recombinant protein.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman (*Cell* 23:175, 1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and

polyadenylation sites may be used to provide the required nontranscribed genetic elements. Each of the foregoing steps can be done in a variety of ways. For example, the desired coding sequences may be obtained from genomic fragments and used directly in appropriate hosts. The construction of expression vectors that are operable in a variety of hosts is accomplished using appropriate replicons and control sequences, as set forth above. The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene and were discussed in detail earlier. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to provide an excisable gene to insert into these vectors.

Other variants on expression vectors include fusion proteins between the gene of interest and other polypeptides. Applications include but are not limited to means of visualization (such as green fluorescent protein, GFP, and variants) or for protein purification (such as polyhistidine, or glutathione-S-transferase, GST).

Specifically contemplated are genomic DNA, cDNA, mRNA and antisense molecules, as well as nucleic acids based on alternative backbones or including alternative bases whether derived from natural sources or synthesized. Such nucleic acids, however, are defined further as being novel and unobvious over any prior art nucleic acid including that which encodes, hybridizes under appropriate stringency conditions, or is complementary to a nucleic acid encoding a protein according to the present invention.

The encoding nucleic acid molecules of the present invention may further be modified so as to contain a detectable label for diagnostic and probe purposes. A variety of such labels are known in the art and can readily be employed with the encoding molecules herein described. Suitable labels include, but are not limited to, biotin, radiolabeled nucleotides and the like. A skilled artisan can readily employ any such label to obtain labeled variants of the nucleic acid molecules of the invention.

Modifications to the primary structure of the nucleic acid molecules by deletion, addition, or alteration of the nucleotide sequence can be made without destroying the activity of the encoded proteins. Such substitutions or other alterations result in proteins having an amino acid sequence falling within the contemplated scope of the present invention.

II. Specific Embodiments

A. G Protein Coupled Receptors

At present, there are about 400 GPCR genes that can be identified from genomic databases, excluding odorant and taste receptors. Ligands for about 200 of these have been identified, leaving the rest as "orphan receptors". The key to uncover the potential of therapeutic benefits of agonists and/or antagonists of these orphan GPCRs is in the ability to identify the natural biological ligands for them to elucidate their biological functions and disease associations. For GPCRs whose ligand is known, the identification of agents that modulate a GPCR-mediated activity allows the development of pharmaceuticals with high affinity and desirable functionality against these receptors for the evaluation of their clinical potential (for a review, see, Debouck and Metcalf, 2000, Annu. Rev. Pharmacol. Toxicol. 40:193-208; Howard *et al.*, 2001, Trends Pharmacol. Sci. 22:132-140).

B. Cyclic Nucleotide-Gated Channels

Cyclic nucleotide-gated (CNG) channels of vertebrates are cation channels controlled by the cytosolic concentration of cGMP and cAMP (for reviews, see Kaupp, 1995, Curr. Opin. Neurobiol. 5:434-442; Finn *et al.*, 1996, Annu. Rev. Physio. 58:395-426; Zogotta and Siegelbaum, 1996, Annu. Rev. Neurosci. 19:235-263; Li *et al.*, 1997, Q. Rev. Biophys. 30:177-193). These channels conduct cation currents, carried by mixed ions–Na+, K+ and Ca^{2+}–and serve to couple both electrical excitation and Ca^{2+} signaling to changes of intracellular cyclic nucleotide concentration. In vertebrate photoreceptors and olfactory sensory receptors, CNG channels depolarize the membrane voltage and determine the activity of a number of Ca^{2+}-regulated proteins involved in cell excitation and adaptation (for reviews, see Kaupp and Koch, 1992, Annu. Rev. Physiol. 54:153-175; Koch, 1995, Cell Calcium 18:314-321).

CNG channels are typically heteromultimers containing homologous α and β subunits. Some CNG channels also have a third subunit as well. For example, a third subunit has been described for the rat olfactory CNG channel (GenBank Acc. No. AF068572). Although they are members of the voltage gated channel superfamily, they are not voltage sensitive, instead responding to changes in cyclic nucleotide concentration. Presently, six human genes have been identified that encode CNG channel subunits. An alignment of the human CNG channels is provided in Figure 8 panels A-C. An alignment of several mammalian CNG channels is provided in Figure 9 panels A and B.

CNG channel subunits typically consist of a cytoplasmic N-terminus, six membrane spanning segments and a cytoplasmic C-terminus. Between the fifth and sixth membrane

spanning segments, a domain critical for pore lining–the P domain–has been identified. Various amino acid residues have been implicated in ion specificity and activation characteristics (see Gavazzo, *et al.*, 2000, *J. Gen. Phys.* 116:311-15, Varnum, *et al.* 1995, *Neuron.*15:619-625).

The rat olfactory CNG channel (CNGA2) forms cAMP-activated channels when heterologously expressed in mammalian cells with a half-maximally effective concentration (EC50) for cAMP of 68 μM (Dhallan *et al* 1990). When co-expressed with CNGB2, or CNGB2 and CNCβ1b (GenBank accession number AF068572) EC50 for cAMP is reduced to 10.3 and 4 μM (Boenigk *et al* 1999). These wild type CNG channels can be used directly for monitoring activation of GPCRs by applying the methods disclosed herein. Wildtype CNG channels of the present invention may also include a third subunit, *e.g.*, the third subunit of rat olfactory CNG channel (GenBank AF06572) or its equivalents in the rat or other species. However, mutants and chimeric constructs of the CNG channels can be used to further increase detectability of GPCR activation. Accordingly, the present invention includes both wildtype and CNG channels having one or more mutations in the following three regions: the cyclic nucleotide binding domain, the C-linker region and the NH_2 terminus that enhance the efficacy of cyclic nucleotide to open CNG channels (Altenhofen *et al* , 1991; Gordon & Zagotta, 1995; Varnum *et al* 1995; Zong *et al*, 1998; Paoletti *et al* 1999; Li & Lester, 1999;Shapiro *et al*, 2000; Scott *et al* 2000; Rich *et al.* 2001; Möttig *et al* 2001; and disclosed herein).

CNG channel subunits of the present invention may be present on a single or on multiple vectors for introduction into a host cell. For example, in the case of a wildtype α/β heteromeric CNG channel, the coding sequences for the α and β subunits may be contained on a single vector which is introduced into the host cell, or they may on separate vectors which are introduced into the host cell either separately or at the same time.

C. G Proteins

Many heterotrimeric G proteins have been cloned, including more than 20 genes encoding various G alpha subunits. The various G subunits have been categorized into six families, on the basis of amino acid sequences and functional homology. These six families are termed G_s, G_i, G_q, G_{olf}, G_o, and G_{12}. With the exception of G_q that results in the release of cytoplasmic Ca^{2+}, all other G proteins mediate their signals through cyclic nucleotides,

primarily cAMP (Watson and Arkinstall, The G-Protein-Linked Receptor Facts Book, Academic Press, London, 1994).

Certain G proteins are considered "promiscuous" G proteins because their G subunits allow them to couple with GPCRs that normally couple with G proteins of other families. For example, two members of the G_q family, human G_{16} and its murine homolog G_{15} are promiscuous G proteins. Although G proteins having these G subunits interact with a variety of GPCRs, they still specifically activate their downstream effector. (See US pat no. 6,004,808 issued to Negulescu ,*et al.*)

D. Host Cells

The present invention further provides host cells that may be transformed with a nucleic acid molecule encoding one or more of a GPCR and/or G protein and/or CNG channel. Host cells can also include cells or cell lines which have an endogenous GPCR and/or G protein which can be used for methods of the present invention in combination with recombinant CNG channel expression. Preferred cells are eukaryotic cells. Eukaryotic cells useful for practicing the present invention include, but are not limited to, mammalian cells. Any cell may be used so long as the cell line is compatible with cell culture methods and compatible with the propagation of the expression vector and expression of the gene product. Preferred eukaryotic host cells include, but are not limited to, yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human cell line. Preferred eukaryotic host cells include Chinese hamster ovary (CHO) cells, for example those available from the ATCC as CCL61, NIH Swiss mouse embryo cells (NIH/3T3) available from the ATCC as CRL 1658, baby hamster kidney cells (BHK), mouse L cells, Jurkat cells, SF9, Xenopus oocytes, 153DG44 cells, HEK cells, PC12 cells, human T-lymphocyte cells and Cos-7 cells, and the like eukaryotic host cells.

Transfection of appropriate cell hosts with a rDNA molecule of the present invention is accomplished by well-known methods that typically depend on the type of vector used and host system employed. With regard to transformation of vertebrate cells with vectors containing rDNAs, electroporation, cationic lipid or salt treatment methods are typically employed, see, for example, Graham *et al.* (*Virol* 52:456, 1973) and Wigler *et al.*, (*Proc Natl Acad Sci USA* 76: 1373-1376, 1979). Similarly, a number of options are commercially available including from Invitrogen/Life Technologies, Promega, Qiagen, etc.

Successfully transformed cells, *i.e.*, cells that contain a rDNA molecule of the present invention, can be identified by well known techniques including the selection for a selectable marker. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the rDNA using a method such as that described by Southern (*J Mol Biol* 98:503, 1975) or Berent *et al.* (*Biotech* 3:208, 1985) or the proteins produced from the cell assayed via an immunological method.

E. Assay Formats

The present invention provides various methods to assay for the presence and/or modulation of GPCR-mediated activity. In preferred embodiments, this may entail the detection of cAMP production by the activation of a CNG channel. In some embodiments, host cells of the present invention are assayed for the influx of Ca^{2+} as a result of their activation by cAMP produced as the result of activation of a GPCR and transduction of the signal through the intermediacy of G proteins and adenyl cyclase to the production of cAMP.

In some embodiments, cells of the present invention may be loaded with a dye that responds to the influx of Ca^{2+} with a change in one or more spectral qualities of the dye. In some embodiments, the dye binds Ca^{2+} directly resulting in an observable change in spectral quality. One example of a dye of this type is fura-2.

In other embodiments, cells may be loaded with dyes that respond to the change in membrane potential that results from the ion flux produced by the activation of the CNG channel. Dyes of this type are known to those skilled in the art (see, Zochowski, *et al.*, 2000, *Biological Bulletin* 198:1-21) and are commercially available, for example, from Molecular Devices, Inc.

CNG channels were proposed as sensors for cAMP in assays aiming to detect Ca^{2+} levels with the calcium sensitive dye Fura-2 (Rich *et al*, 2000, J. Gen. Physiol. 116:147-161). A large number of mutants of a CNG channel alpha subunit have been identified that include C460W (Gordon *et al.*, 1997, Neuron 19:431-441), E583M (Varnum *et al.*, Neuron 15, 619-925), and Y565A change (Li and Lester, 1998, Mol. Pharmacol. 55:873-882). While the mutants enhanced the CNG channel's sensitivity to cAMP, the improved sensitivities are still not sufficient for use in a multiwell format. In the best case so far reported, it required 3-4 X 10^6 cells for the elevated Ca^{2+} level in response to cAMP induction to be detected by a

spectrofluorimeter (Rich *et al*, 2001, J. Gen. Physiol. 118:63-77). In contrast, a typical multiwell assay will involve the use of about 20-50,000 cells per well which is about 100 fold fewer cells than required for Ca^{2+} sensitive fluorescence dyes.

Voltage sensitive dyes that may be used in the assays and methods of the invention have been long used to address cellular membrane potentials (for review, see Zochowski *et al.*, Biol. Bull. 198:1-21). Several classes of fluorescent dyes were developed that include carbocyanine, rhodamine, oxonols and merocyanine that can be obtained from Molecular Probes (Eugene, OR). The three bis-barbituric acid oxonols, often referred to as DiBAC dyes, form a family of spectrally distinct potentiometric probes with excitation maxima at approximately 490 nm (DiBAC4(3)), 530 nm (DiSBAC2(3)) and 590 nm (DiBAC4(5)). The dyes enter depolarized cells where they bind to intracellular proteins or membranes and exhibit enhanced fluorescence and red spectral shifts (Epps *et al.*, 1994, Chem. Phys. Lipids 69:137-150). Increased depolarization results in more influx of the anionic dye and thus an increase in fluorescence. DiBAC4(3) reportedly has the highest voltage sensitivity (Brauner *et al.*, Biochim. Biophys. Acta. 771:208-216). Similar assays were developed for membrane potential assays in high throughput platforms such as FLIPR (Molecular Devices, Sunnyvale, CA). As cAMP also induces Na+ and K+ flux in addition to Ca^{2+} changes of membrane potential as the result of Na+ and K+ flux in the presence of CNG channels can be used as the indicators of intracellular cAMP accumulation.

Detection of the alteration in the spectral characteristics of the dye may be performed by any means known to those skilled in the art. In preferred embodiments, the assays of the present invention are performed either on single cells using microscopic imaging to detect changes in spectral–*i.e.*, fluorescent– properties or are performed in a multiwell format and spectral characteristics are determined using a microplate reader.

One suitable configuration for single cell imaging involves the use of a microscope equipped with a computer system. ATTO's Attofluor® RatioVision® real-time digital fluorescence analyzer from Carl Zeiss is a completely integrated work station for the analysis of fluorescent probes in living cells and prepared specimens (ATTO, Rockville, MD). Calcium can be visualized in real time. The system can observe these ions either individually or simultaneously in combinations limited only by the optical properties of the probes in use. The standard imaging system is capable of performing multiple dye experiments such as Fura-2 (for calcium) combined with GFP (for transfection) in the same cells over the same period of time. Ratio images and graphical data from multiple dyes are displayed on line.

When the assays of the invention are performed in a multiwell format, a suitable device for detecting changes in spectral qualities of the dyes used is multiwell microplate reader. Suitable devices are commercially available, for example, from Molecular Devices (FLEXstation™ microplate reader and fluid transfer system or FLIPR® system). These systems can be used with commercially available dyes such as Fluo-3, Fluo-4, and Calcium Green-1. All of these indicators excite in the visible wavelength range.

The Molecular Devices' FLIPR Fluorometric Imaging Plate Reader (Molecular Devices, Sunnyvale, CA) has been used in a high throughput screening assay to detect transient calcium release from intracellular with a calcium sensitive fluorescent dye in response to the activation of the Gq coupled subclass of receptors that activate the phopholipase signaling pathway. Promiscuous G proteins were used for other GPCRs with mixed results. Until the present invention, there was no comparable assay for cAMP that produces real-time, kinetic information on GPCR receptor activation. Furthermore, there was no easy way to directly examine cAMP accumulation in single cell activated by GPCR ligand in live cells in an imaging platform.

In some embodiments of the present invention, the cells of the invention may be treated with compounds designed to increase the intracellular level of cAMP. For example, the cell may be treated with a "caged" cAMP analogue that can be released in response to photons of light. (see Corrie, *et al. Bioorganic Photochemistry* vol 2 pp 243-305, Wiley and Sons, Chichester, UK, and Hagen, *et al*, 1996, *Biochemistry* 35:7762-7771)

F. Methods to Identify Agents that Modulate GPCR-mediated Activity

An additional embodiment of the present invention provides methods for identifying agents that modulate a GPCR-mediated activity. Agents that bind to the proteins involved in the activity or that affect the expression of these proteins may or may not affect the function of said proteins. Investigation of functional effects of agents includes but is not limited to: 1) effects on ligand binding, 2) effects on G protein coupled signaling pathways, 3) activation or inhibition of receptor down regulation/desensitization.

In one embodiment of the invention, the materials and methods described may be used to identify ligands for a GPCR. This embodiment will be useful to identify ligands for "orphan receptors" *i.e.*, those receptors for which a ligand has yet to be identified. Ligands may be identified by contacting a cell of the invention with a compound that is a putative ligand. The cell may be transfected with a nucleic acid that expresses a GPCR of interest and

optionally at least a nucleic acid that expresses a CNG channel, including one that has been mutated to increase its sensitivity to cAMP. Activation of the GPCR is assayed by measuring activation of the CNG channel. For example, a cell may be loaded with a calcium sensitive dye and/or a voltage sensitive dye and changes in the spectral characteristics of the dye in the presence and absence of the putative ligand may be determined. A compound is identified as a ligand if it induces opening of the CNG channel and a concomitant change in the spectral characteristics of the dye.

In another embodiment of the invention, the ability of an agent to modulate GPCR-mediated activity by, for example, altering ligand binding, may be determined. Alteration of ligand binding may be assessed by the ability of the agent being tested to modulate the binding of a known ligand for the target GPCR. This may be accomplished using the assays described above wherein the GPCR transfected into the cell has a previously identified ligand. Alternatively, an endogenous GPCR with an identified ligand may be used. The ability of the previously identified ligand to induce activity is assayed in the presence and absence of the agent. An agent modulates a GPCR-mediated activity when the activity in the presence of the agent differs–is greater, lesser or of differing kinetic characteristics–from the activity in the absence of the agent. Standard methods of data analysis such as inhibition curves are employed to analyze effects of the agents being tested.

Alteration of activation of G protein coupled signaling pathways requires the presence of an active receptor coupled to a G protein-dependent signaling system. As an example, this may be accomplished by preparing cell lines co-transfected with the GPCR along with a promiscuous G protein such as G α16. This G protein acts as a universal adapter and, when activated by a GPCR partner, results in calcium mobilization (Marchese *et al.*, *Trends Pharmacol Sci*, 20:370-375, 1999). Calcium mobilization, in turn is easily assessed by use of the assays described above. For example, a number of fluorescent intracellular calcium probes are available from Molecular Probes, Inc. Changes in intracellular calcium concentration result in changes in fluorescence intensity and/or characteristics of the probe and may be detected using a fluorescence plate reader according to the manufacturer's instructions. Confirmation that an agent affects G protein-coupled signaling by the receptor is then obtained by incubating cells in the presence of the agent of interest at a suitable concentration–typically between about 10 pM and 1 mM–and determining the resultant changes in intracellular calcium concentration. Standard dose-response curves are generated and analyzed.

G. Uses for Agents that Modulate GPCR-mediated Activity

Agents that modulate one or more GPCR-mediated activities, such as agonists or antagonists of a GPCR, may be used to modulate processes associated with GPCR function and activity. In some embodiments, agents that modulate a GPCR-mediated activity–increase, decrease, or change the kinetic characteristics of the activity–may be used to modulate biological and pathologic processes associated with one or more GPCR-mediated activity.

As used herein, a subject can be any vertebrate, preferably a mammal, so long as the vertebrate or mammal is in need of modulation of a pathological or biological process mediated by a GPCR protein of the invention. The term "mammal" is defined as an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human subjects.

Pathological processes refer to a category of biological processes that produce a deleterious effect. For example, a particular GPCR-mediated activity or level of activity may be associated with a disease or other pathological condition. As used herein, an agent is said to modulate a pathological process when the agent reduces the degree or severity of the process. For example, a GPCR-mediated activity may be associated with a G-protein signaling disorder, such as those associated with other receptors for biogenic amines (see Background section above for examples).

The agents of the present invention can be provided alone, or in combination with other agents that modulate a particular pathological process. For example, an agent of the present invention can be administered in combination with other known drugs. As used herein, two agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

The agents of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

H. Agents that Modulate GPCR-mediated Activity

Potential agents can be screened to determine if application of the agent modulates a GPCR-mediated activity. This may be useful, for example, in determining whether a particular drug is effective in treating a particular patient with a disease characterized by an aberrant GPCR-mediated activity. In the case where the activity is affected by the potential agent such that the activity returns to normal or is altered to be more like normal, the agent may be indicated in the treatment of the disease. Similarly, an agent that induces an activity that is similar to that expressed in a disease state may be contraindicated.

According to the present invention, a GPCR with an identified ligand may be used as the basis of an assay to evaluate the effects of a candidate drug or agent on a cell, for example on a diseased cell. A candidate drug or agent can be screened for the ability to modulate an activity mediated by the GPCR, for example Ca^{2+} influx.

Assays to monitor the modulation of a GPCR-mediated activity may utilize any available means of monitoring for changes in CNG activity, but is preferably accomplished using one or more of the assay formats described above.

Agents that are assayed in the above methods can be randomly selected or rationally selected or designed. As used herein, an agent is said to be randomly selected when the agent is chosen randomly without considering the specific sequences involved in the association of the a protein of the invention alone or with its associated substrates, binding partners, etc. An example of randomly selected agents is the use a chemical library or a peptide combinatorial library, or a growth broth of an organism.

As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a nonrandom basis which takes into account the sequence of the target site and/or its conformation in connection with the agent's action. Agents can be rationally selected or rationally designed by utilizing the peptide sequences that make up these sites. For example, a rationally selected peptide agent can be a peptide whose amino acid sequence is identical to or a derivative of any functional consensus site.

The agents of the present invention can be, as examples, peptides, small molecules, vitamin derivatives, as well as carbohydrates, lipids, oligonucleotides and covalent and non-covalent combinations thereof. Dominant negative proteins, DNA encoding these proteins, antibodies to these proteins, peptide fragments of these proteins or mimics of these proteins may be introduced into cells to affect function. "Mimic" as used herein refers to the modification of a region or several regions of a peptide molecule to provide a structure chemically different from the parent peptide but topographically and functionally similar to

the parent peptide (see Grant, (1995) in Molecular Biology and Biotechnology Meyers (editor) VCH Publishers). A skilled artisan can readily recognize that there is no limit as to the structural nature of the agents of the present invention.

I. Compositions Comprising Agents that Modulate GPCR-mediated Activity

Compositions comprising the agents of the present invention can be provided alone, or in combination with other compositions and/or agents that modulate a particular pathological process. For example, an agent of the present invention can be administered in combination with other known drugs. As used herein, two agents are said to be administered in combination when the two compositions and/or agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

Compositions comprising the agents of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

The present invention further provides compositions containing one or more agents that modulate a GPCR-mediated activity. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise 0.1 to 100 μg/kg body wt. The preferred dosages comprise 0.1 to 10 μg/kg body wt. The most preferred dosages comprise 0.1 to 1 μg/kg body wt.

In addition to the pharmacologically active agent, the compositions of the present invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries that facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient.

Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

The compositions of the present invention can be utilized *in vivo*, ordinarily in mammals, such as humans, sheep, horses, cattle, pigs, dogs, cats, rats and mice, or *in vitro*.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

Example 1

Single Cell Imaging Assay

Figure 2 shows the results of a single cell imaging assay. In this example, HEK293 cells transiently transfected with a CNG channel (SEQ ID NO:3) for two days were loaded with a calcium fluorescent dye prior to the recordings. Specifically, cells were cultured on a microscope Fisher brand cover glass #1 pre-coated with MATRIGEL (Becton Dickinson, San Jose, CA) incubated in culture medium (DMEM with 10% fetal bovine serum) containing 5 μM fura-2 AM (Molecular Probes, Sunnyvale, CA) for 0.5 hour at 37 °C. Calcium fluorescence recordings were made on Attofluor® RatioVision®, a real-time fluorescence imaging device (ATTO, Rockville, MD). This system is capable of performing experiments using multiple fluorescent probes such as Fura-2 (for calcium) combined with GFP (transfection marker) in the same cells over the same period of time. Ratio images and graphical data from multiple dyes are displayed on line. This example demonstrates that activation of Gs-coupled GPCR and adenylyl cyclase can be detected by monitoring a change in cytosolic free calcium concentration in a single cell expressing of CNG channels in real

time. Upon addition of norepinephrine (NE) and forskolin (forsk) at the times indicated by arrows in Figure 2, cytosolic calcium concentration started to rise in GFP-positive–*i.e.*, transfected–cells. Two representative images were taken before (Panel A) and after (Panel B) adding NE showing individual cell responses. Calcium fluorescence changes are averaged respectively in GFP-positive cell population and whole cell population and displayed graphically (Panel C).

Example 2

Comparison of Calcium Sensitive Dyes and Voltage Sensitive Dyes in a Multiwell Format

In this example, recordings were made using a microplate reader FLEXstation (Molecular Devices, Sunnyvale, CA) and the protocols provided with the assay kit were adopted for both the calcium assay and the membrane potential assay. Figure 3 shows the results of a multiwell assay using a mutated CNG channel (SEQ ID NO 5), which is reported to have a lower value EC50 value for cAMP (Rich, *et al.* 2001, *J. Gen. Phys.* 118:63-77) than that of SEQ ID NO:3, and therefore expected to be more sensitive to cAMP change. Figure 3A shows the response to isoproterenol (agonist of β2 adrenergic receptor) determined using a Ca^{2+} sensitive dye, fluo-4 in cells transiently transfected with the CNG channel. There was no significant change in fluorescence of the dye after stimulating cells with a saturating dose of isoproterenol of 10 μM. Figure 3B shows the results of activation with carbachol, a muscarinic receptor agonist, as a positive control for mobilization of intracellular calcium stores via the Gq pathway. The changes in intracellular Ca^{2+} concentration as a result of this treatment were observable.

To establish the utility of CNG channels and membrane potential dyes in detecting intracellular cAMP, forskolin, an adenylyl cyclase activator, was used to generate intracellular cAMP. HEK293 cells transiently transfected with a CNG channel (SEQ ID NO 5) were loaded with the voltage-sensitive dye at room temperature for about 0.5 hour. Figure 4 shows that upon the addition of forskolin, the intracellular cAMP can be readily detected in the presence of a voltage-sensitive dye by using a microplate reader.

Example 3

Assay for Intracellular cAMP in response to GPCR activation Using Mutant CNG Channel with a Membrane Potential Dye

Figure 5 shows the results of a similar assay in which the voltage-sensitive dye of the membrane potential assay kit was used (Molecular Probes, Sunnyvale, CA). DPBS (divalent-free Dulbecco's Phosphate Buffer Salts) supplemented with 0.1 mM $MgCl_2$, 1 mM EGTA and titrated to pH 7.3 was used to reconstitute the voltage-sensitive dye instead of the buffer solution supplied in the commercial kit. HEK293 cells transiently transfected with a CNG channel (SEQ ID NO 5) were loaded with the voltage-sensitive dye at room temperature for about 0.5 hour. A readily detectable change in fluorescence signal was seen at concentrations as low as 0.1 μM isoproterenol that activate β adrenoceptor, as compared to no detectable change with 10 μM isoproterenol using calcium-sensitive dye shown in Figure 3A. Similarly, Figure 6 shows the results with membrane potential dye with a different CNG channel mutant (SEQ ID NO 7).

Example 4

Assay for Intracellular cAMP with Co-expressing GPCR and CNG Channel with a Membrane Potential Dye

The assays of the present invention can be conducted using an exogenous introduced GPCR. DNA encoding a dopamine type I receptor was co-transfected into cells with a mutated CNG channel (SEQ ID NO 7) into HEK273 cells. Figure 7 shows the results of activation of the receptor with its natural ligand, dopamine. Dosage dependent fluorescence signals are obtained immediately following the additional of dopamine in the presence of a membrane potential dye (Molecular Probes, Sunnyvale, CA).

Example 5

Identification of Ligands for an Orphan GPCR in Transiently Transfected Cells

Genes encoding for a wild type or mutated CNG channel protein and a GPCR of interest can be transfected into target cell using standard transfection techniques (Ausuebl *et al.*, Current Protocols in Molecular Biology, (2001) John Wiley & Sons). Two days after transfection, approximately 50,000 cells/well for a 96-well plate and 10,000 cells/well for a 384-well plate may be used to create a confluent cell monolayer with a plating volume of 100 μL/well for 96-well plates or 25 μL/well for 384-well plates.

Cell plates may be removed from the incubator after overnight incubation. An equal volume of Loading Buffer with a membrane potential dye (Molecular Devices, Sunnyvale, CA) can be added to each well (100 μL per well for 96-well plates, 25 μL for 384-well

plates) and the cell plates further incubated for 30 minutes at 37 °C. After incubation, the plates can be directly assayed using a FLIPR or FlexStation.

Candidate natural synthetic ligand collections can be obtained and diluted to concentrations ranging from 1 nM to 10 μM for testing. Membrane potential assays will be performed immediately following the addition of the compounds as described in the FLIPR system manual for membrane potential assay (Molecular Devices, Sunnyvale, CA). Such assays can also be performed in the presence of Ca^{++} sensitive dye (Molecular Devices, Sunnyvale, CA).

Example 6

Identification of Agents that Modulate GPCR-mediated Activity

Compounds may be screened for their ability to function as agents for the modulation of one or more GPCR-mediated activities. A cell prepared according to the present invention may be contacted with a compound and one or more GPCR-mediated activities may be assayed. As an example, stable cell lines expressing a genes encoding for a CNG channel protein and a GPCR of interest can be obtained (Ausuebl *et al.*, Current Protocols in Molecular Biology, (2001) John Wiley & Sons). The GPCR gene can be of either endogenous or exogenous sources. Approximately 50,000 cells/well for a 96-well plate and 10,000 cells/well for a 384-well plate can be used to create a confluent cell monolayer with a plating volume of 100 μL/well for 96-well plates or 25 μL/well for 384-well plates.

Cell plates can be removed from the incubator after overnight incubation. An equal volume of Loading Buffer with a membrane potential dye (Molecular Devices, Sunnyvale, CA) is then added to each well (100 μL per well for 96-well plates, 25 μL for 384-well plates) and the cell plates further incubated for 30 minutes at 37 °C. After incubation, the plates can be directly assayed using the FLIPR. Libraries of compounds can be obtained and diluted to concentrations ranging from 1 nM to 10 μM for testing. Membrane potential assays are performed immediately following the addition of the compounds as described in the FLIPR system manual for membrane potential assay (Molecular Devices, Sunnyvale, CA). Such assay can also be performed in the presence of both membrane potential and Ca^{2+} sensitive dyes (Molecular Devices, Sunnyvale, CA).

Example 7

A homogeneous, kinetic assay with HEK 293-CNG cells in 384-well plates with membrane potential dye

Populations of cells are stably transformed to express CNG channel and established under either adherent or suspension culture conditions. The cells are harvested and adjusted to 1 x 10^6 cells/ml in DMEM (high glucose) comprising 10% FBS. 20 µl of the cell suspension is dispensed per well into 384-well microplates (Corning; 3712) and incubated 16-24 hours prior to assay. Immediately prior to assay, wells of the cell plates are observed microscopically to confirm the presence of confluent lawns of consistently spread cells.

Membrane potential dye stock (Membrane potential reagent kit, Component A, Molecular Devices, R-7056) stock solution is prepared by dissolving one bottle of dye in the kit in 10 ml Dulbecco's Phosphate Buffered Saline (DPBS) supplemented with 20 mM HEPES (pH 7.0), aliquoted into 1 ml portions and stored at –80°C. Dye Loading Buffer is prepared on the day of the assay by diluting 1 ml dye stock with 9 ml of the DPBS supplemented with HEPES at 20 mM, pH 7.0 per 384-well plate. 20 µl Dye Loading Buffer per well is added to the 384-well cell plates. Plates are incubated at room temperature, about 20-25°C, for 1-7 h. During incubation, dilutions of test compounds are prepared in Compound Buffer (10 mM EGTA in Dye Loading Buffer; pH 7.2).

Dye loaded cell plates are then loaded into a FLIPR384, FLEXstation, or other fluorescence microplate reader and assayed per fluorescence microplate reader instructions. For example, in a FLIPR384, 488 nm excitation and 540-590 nm emission filters are used; for FLEXstation and other fluorescence microplate readers, wavelengths close to the maxima of absorption and emission of the dye are used: for example, 540 nm excitation and 560 nm emission for the membrane potential dye of Molecular Devices, R-7056). Ten µl of test compound in Compound Buffer is added per well and the results are recorded.

In the present example, the CNG channel assay was adapted to a HTS platform, FLIPR (Molecular Devices). Specifically, a stably transformed HEK 293H cell line expressing CNG channels (SEQ ID NO: 7) was used for a cAMP assay. Cells were seeded into 384-well plate coated with MATRIGEL (Becton Dickinson, 354234) using a Multidrop 384 dispenser (Titertek Instruments, Inc.). Well-to-well variability of CNG channel assay for cAMP responses was assessed in the recordings of 4 min duration shown in Fig. 10. 30 seconds after beginning the recordings, Isoproterenol (1 µM final) was added to the wells of columns 1-12 while compound buffer was added as a control to the wells of columns 13-24 at

time of (Fig. 10). HEK293H-CNG cell line and the parental cell line lacking CNG was used in Fig. 10A and 10B respectively. Dose-dependent responses to isoproterenol were obtained in separate recordings. Multiple fluorescence traces in response to various doses of isoproterenol were overlaid (Fig. 10C). Data consistency is demonstrated in Fig. 10D by overlaying multiple responsive curves for 1 μM isoproterenol from the plate of Fig 10A.

Example 8

A kinetic assay with HEK 293H-CNG cells with calcium-sensitive dye

Populations of cells, *e.g.*, HEK 293 or HEK 293H, are stably transformed to express CNG channel, *e.g.*, SEQ ID NO: 7, and established under either adherent or suspension culture conditions. The cells are harvested and adjusted to 1 x 10^6 cells/ml in DMEM (high glucose) comprising 10% FBS. 20 μl of the cell suspension is dispensed per well into 384-well microplates, or 100 μl of the cell suspension is dispensed per well into 396-well microplates, and incubated 16-24 hours prior to assay. Immediately prior to assay, wells of the cell plates are observed microscopically to confirm the presence of confluent lawns of consistently spread cells.

Calcium-sensitive dye Fluo-4 AM (Molecular Probes, F-14202) is prepared as a 4 mM stock solution in DMSO and stored at –20°C. On the day of assay, stock solution is diluted to a final dye solution concentration of 4 μM cells in Hanks' Balanced Salt Solution (HBSS, pH 7.2). Cells are loaded with the dye by replacing the DMEM +FBS in the wells with the HBSS dye solution and incubation at room temperature for 1 hour. During incubation, prepare compound plates. Test compounds are dissolved in HBS or HBS supplemented with 10 mM $CaCl_2$.

Dye loaded cell plates are then loaded into a FLIPR384, FLEXstation, or other fluorescence microplate reader and assayed per fluorescence microplate reader instructions.

In the present example, a stably transformed HEK 293H cell line expressing CNG channels (SEQ ID NO: 7) was assayed for calcium uptake. 30 seconds after beginning the recordings, isoproterenol was added to wells at final concentrations of 0.3, 1.0, 3.0, 10.0, 30.0 and 300.0 nM. Buffer solution only was added to control wells. Dose-dependent responses to isoproterenol were recorded and multiple fluorescence traces in response to the various doses of isoproterenol were overlaid as shown in Fig. 11.

Example 9

Single Cell Imaging Assay:

In this example, stably transformed HEK 293H cells expressing a CNG gene (SEQ ID NO: 7) were assayed using a voltage-sensitive dye (Molecular Devices, R-7056). Cells were seeded into a 96-well plate pre-coated with MATRIGEL. Membrane potential fluorescence recordings were made on Pathway HT imaging platform (ATTO, Rockville, MD) in the same cells over the same period of time. Confocal fluorescence intensity images were displayed in Fig. 12 with an arbitrary intensity range of 280-650. The images were obtained before and 15, 30 and 45 seconds after addition of isoproterenol to final 1 μM in a time sequence marked in the Fig 12A. One imaging area of 50x50 um was displayed in Fig 12A. Average of fluorescence traces obtained in 71 imaged cells was shown in Fig. 12B. The time of addition of isoproterenol was marked with an arrow.

Example 10

Assay for intracellular cAMP using CNG channels of wild type subunits and subunits containing mutations

Relative fluorescence responses to cAMP rise of in cells transiently transfected with wild type α + β subunit heteromeric CNG channels, homomeric CNG channel α subunits comprising various mutations, or homomers of CNG channel wild-type α subunit alone was explored. In this example, HEK293 cells were transfected two days prior to the recordings with rat olfactory wild type CNG channel α subunit (NCBI LocusID 25411, SEQ ID NO 1) plus β subunit (NCBI LocusID 85258), the rat olfactory CNG channel α subunit containing mutations C460R/E583M, C460H/E583M, C460W/Y565A /E583M (SEQ ID NO 7), or Y565A (SEQ ID NO 3), and α subunit alone (NCBI LocusID 25411, SEQ ID NO 1). The cells were incubated in *Dye Loading Buffer* containing membrane potential dye (Molecular Devices, R-7056) at room temperature. Isoproterenol was dissolved in Compound Buffer as in Example 8 to a final concentration of 300 nM and added at the time marked with an arrow (Fig. 13). Figure 13 shows that isoproterenol responses in cells expressing heteromeric CNG channels composed of wild type α and β subunits are larger than or similar to those expressing CNG channels formed by homomeric wild type α subunit or α subunit homomers containing mutations of C460R/E583M, C460H/E583M, C460W/Y565A /E583M, or Y565A (Fig. 13).

Example 11

Sensitivity Comparison between CNG Channel Assay with a Conventional Transcription Assay and a cAMP ELISA Assay

A number of cAMP assay technologies have been developed based on the principles of competitive binding of cAMP antibody or transcription of genes regulated by cAMP-response elements (CRE). In assays using cAMP-specific antibodies, cell lysis is required to release cAMP to the assay media. As a result, assay sensitivity is compromised as cell number is reduced. In gene reporter assays, more false positive and negative recordings are expected as CRE transcription can be affected non-specifically by varieties of signaling pathways. In contrast, the CNG channel assay provides a direct physiological readout of cAMP intracellularly to avoid the problems associated with conventional indirect cAMP assay technologies. CNG channels are targeted to the plasma membrane and co-localized with adenylyl cyclase to permit a sensitive detection of a local cAMP rise. Because fluorescence readout in the CNG channel assay derives from activity of single live cells, assay sensitivity is not compromised by reducing cell numbers, as it may be in indirect assays.

A comparison of the CNG channel assay was made with an ELISA-based anti-cAMP antibody binding assay (Amersham Biotrak kit, used according to kit directions) and a conventional CRE-Luciferase gene reporter assay in 96-well format. Dose-response curves were generated to forskolin using the same ligand concentrations for all 3 assay formats. Fig 14 shows that the response curve is left-shifted in general using the CNG Channel assay and demonstrates that low concentrations of forskolin induced a significantly larger response in CNG channel assay than in the ELISA and gene reporter assay formats, indicating that CNG channel assay is more sensitive than these conventional cAMP assays.

Example 12

CNG Channel Assay for Gs-Coupled GPCRs of Different Families

To show the sensitivity of the CNG channel assay as a kinetic assay for Gs-coupled GPCRs, Gs-coupled GPCRs were randomly chosen from class B (the secretin family) and class A (including, for example, biogenic amines, peptides, prostanoids, and adenosine receptors) for assay. Activation of the GPCRs listed in Table 1 by their cognate agonists was examined applying CNG channel assay. All of these GPCRs were read out successfully by CNG channel assay, demonstrating the ability of the CNG assay to provide accurate kinetic measurements of Gs-coupled CPCR activation, regardless of its ligand family. Fig. 15

showed dose-response curves of three GPCRs tested to illustrate their relevance to the other pharmacological analysis.

Table 1. Gs-coupled GPCRs tested with CNG channel assay

Receptor	Ligand Tested	Ligand Type
Dopamine D1	Dopamine	Mono amine
Beta-Adrenergic	Isoproterenol, Epinephrine	Mono amine
Histamine H2	Histamine	Mono amine
5-Hydroxytryptamine 4	5-Hydroxytryptamine	Mono amine
Tyramine	Tyramine	Mono amine
Prostaglandin E 2	Prostaglandin E1	Lipid
Prostaglandin D2	Prostaglandin D2	Lipid
Calcitonin	Calcitonin	Peptide
Glucagon	Glucagon	Peptide
Parathyroid Hormone 1	PTH, PTHrP	Peptide
Vasoactive Intestinal Peptide 1	VIP	Peptide
Arginine Vasopressin 2	Arginine Vasopressin	Peptide
Melanocortin 1	α MSH	Peptide
Melanocortin 3	α MSH	Peptide
Melanocortin 4	α MSH	Peptide
Melanocortin 5	α MSH	Peptide
Adenosine A2b	NECA	Nucleotide

Example 13

Robustness of CNG channel assay in comparison with assay using promiscuous G protein and G protein chimera

Calcium fluorescence assays using the promiscuous G protein $G\alpha_{16}$ and the G protein chimera $G\alpha_{qs}$ have been previously used to measure intracellular calcium rise. However, measurement of GPCR activation using either $G\alpha_{16}$ or $G\alpha_{qs}$ is indirect, as both re-direct the activity of some Gs-coupled GPCRs to a phospholipase C-mediated intracellular calcium rise. Because the coupling efficiency of $G\alpha_{16}$ and $G\alpha_{qs}$ varies between Gs-coupled GPCRs, the final calcium signal readout varies between receptors.

In this example, activation of the tyramine receptor was examined using the CNG channel assay and compared with calcium fluorescence assays using $G\alpha_{16}$ and $G\alpha_{qs}$. Similar amounts of plasmids comprising CNG channel, $G\alpha_{16}$ or $G\alpha_{qs}$ were used for transient

expression in HEK293 cells also transiently expressing tyramine receptor. Calcium assays were performed following the protocol provided by Molecular Devices. For each concentration of tyramine, three recordings were made to obtain an average response in cells expressing $G\alpha_{16}$, $G\alpha_{qs}$ and CNG channel respectively. As shown in Fig. 16, tyramine receptor activation is detectable using the CNG channel assay with membrane potential dye, but not by calcium fluorescence assays using $G\alpha_{16}$ or $G\alpha_{qs}$ and calcium dye (Fig. 16).

Example 14

Identification of Agonists and Antagonists of GPCRs Applying CNG Channel Assay

The CNG channel assay can be used to identify GPCR ligands. In this example, the CNG channel assay was used to probe a panel of adrenergic compounds for those that are agonists or antagonists of β-adrenoreceptors. Stably transformed HEK293H cells expressing a CNG channel gene (SEQ ID NO: 7) were seeded into 96 well plates and grown as described in previous examples. Test compounds were arrayed in columns 1-11 of a 96-well plate as shown in Fig. 17B, with buffer only as a control in column 12. Test compounds were added to the cell plates 20 seconds after the start of recordings to a final concentration of 1 μM. Isoproterenol, 10 μM final concentration, was added at 120 seconds to evoke cAMP rise. Time duration of recordings was 230 seconds. Agonists were identified by the detection of a fluorescence rise immediately following the addition of the test compound, before the addition of isoproterenol and are marked by hollow circles in Fig. 17A. Antagonists were identified by delaying or ablating the response of cells to isoproterenol stimulation, as marked by solid squares in Fig 17A.

Example 15

Endpoint Assay

The CNG channel assay can also be used to perform endpoint assays. Decay of fluorescence responses results from desensitization of CNG channel was effectively removed by chelating extracellular calcium by EGTA and supplementing inhibitors of phosphodiesterase. Stably transformed cells expressing a CNG channel gene were seeded into 384 well plates and grown as described in previous examples. Forskolin was dissolved at a final concentration of 30 μM in Compound Buffer containing EGTA. Fluorescence intensity values of cells incubated with forskolin or with Compound Buffer only were read

using FLEXstation (Molecular Devices) at different time points after forskolin stimulation. Treatment with forskolin resulted in a fluorescence intensity of 5.3±0.5 x 10^5 RFU (30 μM forskolin, n=192) at 90 minutes after stimulation, versus 1.9±0.1 x 10^5 RFU (buffer control, n=192), representing a 2.8-fold increase.

Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents, patent applications and publications referred to in this application are herein incorporated by reference in their entirety.